Xist-mediated chromatin changes that establish silencing of an entire X chromosome in mammals

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Abstract

X chromosome inactivation (XCI) ensures an equal gene dosage between the sexes in placental mammals. Xist, a modular multi-domain X-encoded long non-coding RNA coats the X chromosome in cis during XCI. Xist recruits chromatin remodelers and repressor complexes ensuring silencing of the inactive X (Xi). Here, we review the recent work focused on the role of Xist functional repeats and interacting RNA-binding factors in the establishment of the silent state. Xist orchestrates recruitment of remodelers and repressors that first facilitate removal of the active chromatin landscape and subsequently direct the transition into a repressive heterochromatic environment. Some of these factors affect silencing on a chromosome-wide scale, while others display gene-specific silencing defects. The temporal order of recruitment shows each silencing step is party dependent on one another. After the Xi is established, many of the factors are dispensable, and a different repertoire of proteins ensure the silenced Xi is maintained and propagated.

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Introduction

Sex chromosomes in Therian mammals evolved from a pair of autosomes (Reviewed in Ref. [1]). During evolution, the Y chromosome lost most of its genes resulting in a gene imbalance between male and female placental mammals. X chromosome inactivation (XCI) is the process via which one of the two female X chromosomes is epigenetically silenced to ensure equal dosage of X-linked genes between sexes [2]. The X inactive-specific transcript (Xist) is a long non-coding RNA encoded on the X-chromosome with a pivotal role in orchestrating this process [3,4]. Xist has a modular structure harboring six conserved repetitive domains with distinct characteristics that expanded its function in evolution (Repeat A till Repeat F) [5−7]. The sequence and conformation of the domains allow for recruitment of a large repertoire of proteins that aid Xist in separate phases of chromosome silencing which have been extensively researched.

Xist-mediated silencing can be divided into three functionally distinct phases: initiation, establishment, and maintenance. The initiation phase starts with determining the X to autosome ratio, leading to female exclusive mono-allelic upregulation of Xist and spreading *in cis* [8,9]. In the establishment phase, active chromatin marks are removed and replaced by repressive marks in a stepwise manner leading to heterochromatinization and CpG island methylation of the Xi [10,11]. In the maintenance phase, Xist remains associated with the inactive X while the silent state is propagated through a near-infinite number of cell divisions. Here, we review new insights in the temporal order of Xist-mediated chromatin changes and factors involved in establishment of the silent state.

Removing the active chromatin landscape requires Xist Repeat A and SPEN

Establishment of the Xi requires spreading of Xist in *cis* and subsequent chromosome-wide repression of transcribed genes [5,9,12]. In mouse, Repeat A in Xist consists of 7.5 highly conserved repetitive motifs that form several hairpin-like structures and loops, required for the initial silencing steps [5,13−16]. Loss of Repeat A results in almost complete absence of silencing. Biallelic X-linked expression persists in the absence of Repeat A, even though Xist still spreads *in cis*. In Repeat A mutants also the active chromatin marks remain present, suggesting repression of active transcription requires Repeat A interactors [17].

Several studies identified SPEN (also known as SHARP) to interact with Repeat A to form a bridge

between repressor complexes and Xist (Figure 1) [18–22]. SPEN is a large multidomain protein, containing four RNA-recognition motifs, a nuclear receptor interaction domain, and a SPEN paralogue/orthologue C-terminal (SPOC) domain, while lacking a chromatin-modifying domain. SPEN interacts with RNAs and mediates silencing in the Notch/RBP-Jκ pathway and of retroviral elements [23–25]. Xist recruits SPEN co-transcriptionally and co-localizes on the X during the inactivation process. CUT&RUN studies indicate enrichment of SPEN with a preference for active enhancers and promoters, while association is lost when silencing is established [21]. The SPOC domain of SPEN is involved in recruitment of several repressor complex members of NR corepressor (NCoR), the silencing mediator of retinoic acid or thyroid hormone receptor (SMRT) and nucleosome remodeling deacetylase (NuRD) complex [21,22,24]. SPEN is essential for XCI and artificial tethering of the SPOC domain to Xist in SPEN deficient or Repeat A mutant cells only partially restores silencing, suggesting other repressors that are required for silencing the Xi potentially bind to SPEN independent of the SPOC domain [21,26].

Repressor complexes mediate deacetylation as a crucial early step in establishing silencing [26]. Enhancer deacetylation in XCI mostly requires HDAC3, as part of the NCoR/SMRT complex. Multiple HDACs are present in mammals, but exclusively loss of HDAC3 affects Xi silencing [17,22]. HDAC3 was suggested to be pre-bound at enhancers in a catalytically inactive state [17]. SPEN potentially activates part of the pre-bound HDAC3 population by delivering NCoR/SMRT complex members, leading to histone deacetylation and facilitating H3K4me1 demethylation [17,21,22,27]. SPEN localization shows overlap with NuRD complex members predominantly at promoters, which are deacetylated and later demethylated in a stepwise manner [17,21]. Ultimately, removal of active marks leads to loss of transcription and decommissioning of enhancers and promoters. Even though these results hint at an exclusive role for SPEN in enhancer and promoter deacetylation through NCoR/SMRT and NuRD, both complexes likely function partially redundantly, which is evident from the HDAC3 knock-out phenotype that to a lesser extent also delays promoter deacetylation [17] and localization of NuRD complex members to enhancer regions [21,28].

SPEN pioneers Xist-dependent recruitment and/or activation of pre-bound repressor complexes required for removing the active chromatin landscape as the initial first step in XCI [17,21]. These changes are essential for recruitment of downstream chromatin writers and erasers that further lock in the silencing process. Interestingly, loss of SPEN after the Xi is established, does not result in Xi reactivation [17]. It is tempting to speculate that Repeat A and SPEN are

Figure 1
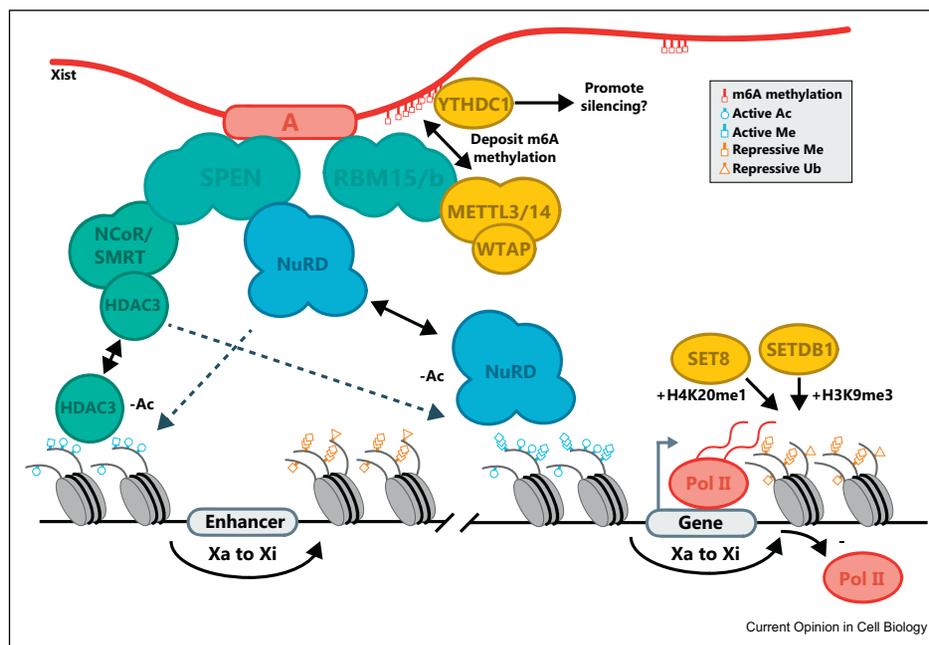
Xist Repeat A facilitates the removal of the active chromatin landscape through the recruitment of SPEN and repressor complexes. SPEN interacts with Xist Repeat A leading to activation and/or distribution of repressor complexes NCoR/SMRT-HDAC3 to enhancers as well as NuRD to promoters. Histone deacetylation leads to demethylation and facilitates enhancer−promoter decommissioning, allowing for deposition of additional repressive marks. RBM15/RBM15b recruit m6A RNA methylation complex members, leading to m6A methylation of Xist and recruitment of YTHDC1.

exclusively required in early establishment of silencing, after which downstream chromatin remodelers and writers stabilize and propagate the silenced state.

Specialized role in silencing for other repressing complexes

The role of additional Repeat A interacting proteins in establishment of the Xi have been up for debate. RBM15 and RBM15b, two SPEN homologues, direct RNA m6A methylation complexes toward Xist [22,29,30]. Many eukaryotic RNAs are regulated by this RNA modification, which is involved in folding, stability, splicing, and recruitment of binding partners (Reviewed in Ref. [31]). Knock-down of RBM15 and m6A methylation complex members WTAP and METTL3 was found to reduce efficiency of Xist-mediated silencing, suggesting that m6A methylation is required for recruitment of proteins involved in silencing [20,30]. Indeed, binding of the m6A-reader YTHDC1 to Xist was lost in the absence of m6A and tethering YTHDC1 to Xist partially rescued the silencing defects observed in Repeat-A mutants as well as RBM15/RBM15b and METTL3 knock-down conditions [26,30]. Other studies report m6A methylation of Xist only plays a minor specialized role, as limited effects on silencing were observed in RBM15-deficient or m6A methylation complex members WTAP- and METTL3-deficient cells [15,22]. The exact role of m6A methylation, YTHDC1 and how they affect chromatin changes in XCI, therefore, needs further investigation.

During Xist accumulation histone modifications associated with gene activity are removed (H3K9ac, H3K27ac, H3K36me3, H4ac) and repressive histone modifications are deposited (H2A119ub1, H3K9me2/3, H3K27me3, H4K20me1) (Figure 2) [17,32−36]. SETDB1, important in catalyzing H3K9me3, is required for silencing a subset of genes exclusively in the establishment phase, while cross-talk with the DNA methylation machinery stabilizes repression in the maintenance phase [37,38]. SET8 (also known as PR-Set7) catalyzes H4K20me1, regulating chromosome

compaction, but the role of SET8 in XCI is unclear [34,39,40]. In *Caenorhabditis elegans* SET8 orthologues catalyze H4K20me1 to affect the chromosome architecture, contributing to dosage compensation where gene expression is reduced by half [41,42]. Also, the nucleosome composition changes to stabilize the Xi as H2A is exchanged with MacroH2A in a cell cycle−dependent manner [43−45]. MacroH2A enrichment is strongest at stably silenced regions mostly in the maintenance phase of XCI. It has been suggested that the structural differences of the MacroH2A linkers inhibits chromatin remodelers, preventing transcription factor binding and promoting heterochromatin formation [46]. Many of the other changes in histone modifications on the Xi are less well understood, and factors involved are not yet identified.

Establishment and maintenance of gene silencing by Polycomb complexes

Polycomb Group (PcG) protein complexes have been implicated to play a crucial role in maintaining silencing of the Xi and altering the organization of the chromosome [35,36]. PcG proteins are conserved in evolution and their expansion during the emergence of vertebrates is thought to accompany the increased complexity of regulating gene expression in a broad range of cellular processes [47,48]. PcG proteins can be divided into two main complexes with several variants. Polycomb Repressive Complex (PRC) 1 catalyzes mono-ubiquitination of H2AK119 while PRC2 catalyzes mono-, di-, and trimethylation of H3K27.

Heterogeneous nuclear ribonucleoprotein K (hnRNPK) acts as a molecular bridge between variant non-canonical PRC1 (vPRC1) and Xist Repeat B and to a lesser extend Repeat C through poly-cytosine-rich repeats [49−51]. The vPRC1 complex containing either PCGF3 or PCGF5 deposits H2AK119ub1, the accumulation of which is partly dictated by Xist spreading [17,49,51]. Xist entry sites, which are gene-dense regions in topological close proximity with the Xist locus, show early accumulation of Xist RNA and overlap with

Figure 2
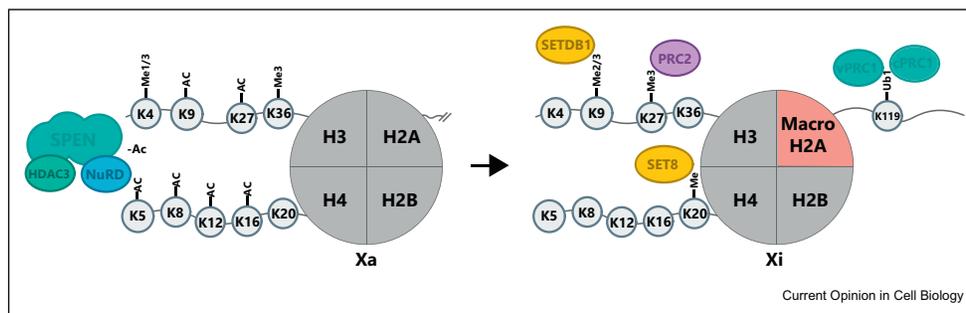
Summary of histon changes occurring in the transition from the active X to the inactive X.

regions pre-marked with PcG-associated histone modifications [12,17,52−54]. Thereafter, proximal intergenic regions accumulate H2AK119ub1 as well as spreading along the X to silenced gene bodies, promoters and the remaining Xi. The vPRC1 complexes interact with H2AK119ub1 through subunits RYBP/YAF2, contributing to spreading [51]. PRC2-subunit JARID2 recognizes H2AK119ub1, recruiting PRC2 and facilitating H3K27me3 deposition [55−57], of which the recruitment to the Xi might be further modulated by Cdk8 kinase activity, a Mediator complex member [58]. Despite following the same patterns, PRC2-dependent H3K27me3 deposition lags behind H2AK119ub1, suggesting H2AK119ub1 comes first and directs H3K27me3 deposition in XCI [17]. In turn, H3K27me3 directs recruitment of canonical PRC1 (cPRC1) that recognizes H3K27me3 through the chromobox-containing protein (CBX), further promoting H2AK119ub1 deposition [59−61]. In a feed-forward loop, deposition and spreading in *cis* of both PcG-marks is stimulated and reinforced, facilitating the transition to a repressive chromatin environment of the Xi (Figure 3). In line with this, recent reports show that vPRC1-mediated deposition of H2AK119ub1 is poorly propagated through multiple cell divisions, which is an absolute requirement for XCI [62]. Therefore, it is likely that silencing in XCI is reinforced by a mutual requirement of cPRC1 as well as PRC2 in facilitating maintenance of Xi silencing.

The interplay of SPEN and PcGs in establishing silencing

Efficient spreading of PcG-marks requires removal of the active chromatin landscape [17]. In the absence of transcriptional silencing in Repeat A mutants, PcG proteins can be recruited, but PcG-associated histone modifications remain mostly restricted to intergenic regions, while spreading into active gene bodies is limited. This observation highlights the notion that PcGs largely act in a secondary step, downstream of Repeat A and SPEN-mediated silencing and PcG-modifications mostly follow rather than direct silencing of target genes as postulated previously [63,64]. This is in line with findings indicating that deacetylation by NuRD and HDAC3 are required for H3K27me3 deposition by PRC2 in gene silencing [17,22,65]. PcG proteins can also be recruited independent of Xist to specific CpG islands of genes containing PcG-associated marks [14,53,66,67]. These CpG islands act as nucleation sites and could allow for limited local accumulation of PcG independent of Xist Repeat B/C but likely require neighbouring genes to be silenced for spreading. Therefore, predominantly Xist-dependent and to a lesser extent independent recruitment of PcGs is instrumental in the polycomb domain formation and stabilizing the Xi.

The importance of Repeat B/C and PcGs in establishing early gene silencing in XCI is still under debate. Loss of

Figure 3
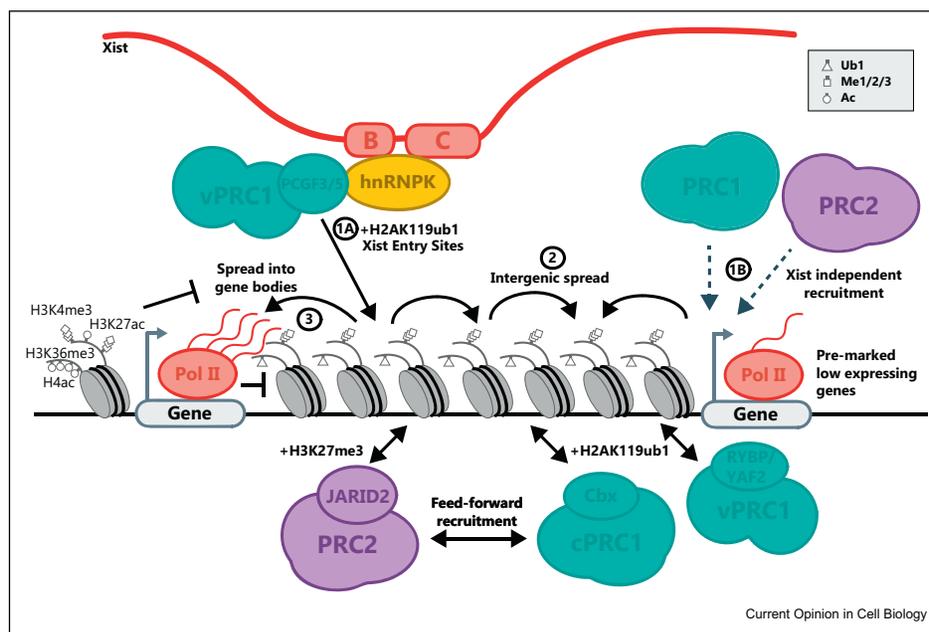
Xist-mediated recruitment of PcG proteins and PcG-mark accumulation. Repeat B/C of Xist recruit vPRC1 through interacting with hnRNPK, resulting in H2AK119ub1 deposition at Xist Entry Sites (1A). PcG-mark accumulation largely follows Xist-spreading, showing enrichment at moderate sites first, spreading mostly to intergenic regions (2). PcG-marks spread into promoters and gene bodies in response to silencing (3). Feed forward recruitment of PRC2 and PRC1 ensures PcG-marks accumulate and facilitate Polycomb-domain formation of the Xi. Alternatively, pre-marked CpG islands act as nucleation sites independent of Xist (1B), leading to accumulation (2).

Xist Repeat B/C and PcG-recruitment has been implicated in loss of silencing efficiency [15,49,66] and reduced Xist accumulation [68]. Other studies indicate that loss of Repeat B is dispensable for the initiation of silencing, exemplified by initial silencing of most X-linked genes, but silencing erodes later in differentiation [14]. A subset of mostly lowly expressed genes, however, are unable to be silenced in absence of Repeat B/C and rely on PcG activity [15]. Moreover, PRC2 knock-out embryos do initiate XCI in the developing epiblast, although loss of PRC2 is lethal at a later stage [69]. This suggests that initiation of silencing of most genes can occur independent of PcG proteins; however, this inactive state is likely transient, poorly maintained, and inefficiently propagated. PcG proteins, therefore, reinforce the silenced X and in turn direct recruitment of downstream proteins that further maintain silencing. This affects the nuclear organization and facilitates DNA methylation (Reviewed in Ref. [70]). PcG proteins, PcG-marks, and Polycomb domain formation, therefore, appear to form the bridge between the transient state of the silenced X and the propagated stably silenced Xi with methylated CpG islands present in somatic cells.

Concluding remarks

XCI serves as an excellent model to unravel gene silencing on a chromosome-wide scale. The different silencing defects observed in Xist Repeat and Xist-interacting protein knock-out studies at various stages of XCI highlight an Xist-directed multi-phasic process requiring a separate orchestra of chromatin-modifying complexes, each dependent on the activity of one another. The temporal order of events show Xist-mediated deacetylation and gene silencing as the crucial first steps. This ultimately allows for Polycomb domain formation and the transition to the heterochromatic Xi. Still, many additional layers of chromatin regulation are unexplored, and histone changes less well understood, providing excellent starting points for future work. More chromatin remodelers and repressor complexes might be recruited in a Xist-dependent matter, while others mostly respond to chromatin changes that occur. Global defects show the generality of factors involved in silencing, as shown by the loss of SPEN and Repeat A [5,15,21], while gene-specific defects highlight requirements for specific repressor complexes, evident from SETDB1-loss [37]. Further insights in XCI will aid in elucidating the missing puzzle pieces of how chromatin remodelers and repressor complexes execute gene silencing chromosome-wide and gene-specifically, as well as how these silencing pathways depend and communicate to one another.

Credit author statement

JB and JG were both involved conceptualization and writing the manuscript.

Conflict of interest statement

Nothing declared.

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** of outstanding interest

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